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FURTHER CHARACTERIZATION OF BACTERIOCINS
PLANTARICIN BN, BAVARICIN MN AND PEDIOCIN A

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ABSTRACT

We report here several characteristics of bacteriocins which are inhibitory to Clostridium botulinum and Listeria monocytogenes. Plantaricin BN produced by Lactobacillus plantarum BN, bavaricin MN produced by Lactobacillus bavaricus MN, and pediocin A produced by Pediococcus pentosaceus 43200 all demonstrated a bactericidal mode of action and retained some activity after heating at 60°C for ten minutes or 100°C for 5 minutes. pH and temperature optima for production on solid media were pH 7.9 and 15°C for plantaricin BN, pH 6.5 and 30°C for bavaricin MN, and pH 6.15-7.9 and 30°C for pediocin A. The molecular weight of plantaricin BN appeared greater than 10,000, that of bavaricin MN 22,600, and that of pediocin A 11,000. All of these bacteriocins were produced during the growth phase of the bacterial cultures.

INTRODUCTION

Minimally processed refrigerated meat products which rely solely on refrigeration for preservation pose a significant health risk. Many foodborne pathogens including Listeria monocytogenes, Yersinia enterocolitica, Aeromonas hydrophila and Clostridium botulinum type E can grow at refrigeration temperatures (Palumbo, 1986, 1987). Secondly, if temperature abused, these foods are particularly susceptible to botulinal growth and toxin production (Nottermans et al., 1990).

Certain lactic acid bacteria produce bacteriocins, proteinaceous antimicrobials. Bacteriocins produced by L. plantarum BN, L. bavaricus MN and P. pentosaceus 43200 are active against psychrotrophic pathogens including L. monocytogenes (Lewus et al., 1991) and against C. botulinum (Okereke and Montville, 1991). Further characterizations of these bacteriocins, important to their commercial application, are reported in this study.

MATERIALS AND METHODS

Bacterial Strains and Growth Media. The bacteriocinogenic strains examined were Lactobacillus plantarum BN and Lactobacillus bavaricus MN, isolated from retail beef (Lewus et al., 1991), and Pediococcus pentosaceus 43200, isolated from cucumber fermentations (Daeschel and Klaenhammer, 1985). Lactococcus lactis ATCC 11454, a nisin producer, was used as a positive control strain. Lactobacillus sake ATCC 15521 was used as the indicator strain in the spot-on-the-lawn, the plate diffusion, well-diffusion and

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SDS-PAGE activity assays (Lewus and Montville, 1991). Stock cultures were maintained at -80°C in 20% glycerol. Working cultures were made as stabs on Lactobacilli MRS broth (Difco, Detroit, MI) prepared with 0.5% glucose and 1.5% Bacto agar (Difco). Strains were maintained as stab cultures and transferred bimonthly for a maximum of six transfers before new working cultures were prepared.

Cidal versus Static Mode of Action. A plate diffusion assay was used to determine cidal versus static mode of action for the bacteriocins by two methods as described by Lewus et al. (1992).

Optimal pH and Temperature for Bacteriocin Production on Solid Media. The pH optima for bacteriocin production was determined as described by Lewus et al. (1992) using 0.2 M MES (2-[N-morpholino] -ethanesulfonic acid, Calbiochem, LaJolla, CA) and 0.2 M HEPES (N-2-hydroxyethyl -piperazine -N'-2-ethanesulfonic acid, Calbiochem). To examine the influence of temperature on bacteriocin production, MRS broth was inoculated with L. plantarum BN, L. bavaricus MN, P. pentosaceus 43200 and L. lactis 11454 and incubated overnight at 30°C. Two ul of the overnight culture was spotted onto the surface of TSAYE plates and incubated anaerobically for four days at 4, 10, 15 and 30°C. The plates were overlaid with BHI 1% agar seeded with 10⁴ to 10⁵ L. sake 15521 per ml and then incubated anaerobically overnight at 30°C. The zones of inhibition on the plates were measured as the distance in mm from the edge of the colony to the edge of the well (Druegeon et al. 1987).

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Determination of Broth Media for Bacteriocin Production. *L. plantarum* BN, *L. bavaricus* MN and *P. pentosaceus* 43200 were inoculated into APT broth, MRS broth, MRS broth supplemented with three levels of Tween 80 (0.1%, 0.5% and 1.0%, Fisher, Springfield, N.J.), MRS broth supplemented with 2.0% yeast extract, BHI broth, BHI broth supplemented with 2.0% yeast extract, Trypticase soy broth without dextrose (BBL) supplemented with 1.0% glucose or 1.0% glucose and 2.0% yeast extract. (Preformulated Trypticase soy broth without glucose supported bacteriocin production when glucose was added, but the same medium prepared from individual ingredients did not.) All tubes were incubated overnight at 30°C. The pH of the broth was then adjusted to 6.5 with 6 N NaOH. The cells were removed by centrifugation at 13,000 x g for 5 min. A well-diffusion assay was performed on the supernatants.

Determination of Growth Phase of Bacteriocin Production in Broth. *L. plantarum* BN and *L. bavaricus* MN were propagated in APT broth overnight at 30°C and *P. pentosaceus* 43200 was propagated in Trypticase soy broth without glucose plus 1% glucose overnight at 30°C. Using a 1% inoculum, a growth curve at 30°C was determined. At regular time intervals, the $A_{60\text{nm}}$ was monitored. Doubling times (t_d) were determined by linear regression of data obtained during the exponential phase of growth. At the same time a sample was withdrawn, the pH was adjusted to 6.5 with 6 N NaOH and cells removed by centrifugation at 13,000 x g for 5 min. All supernatant samples were stored at 4°C overnight and

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analyzed the next day for bacteriocin production by the well-diffusion assay.

Preparation of Crude Bacteriocin. *L. plantarum* BN was propagated overnight in Brain heart infusion broth (Difco) supplemented with 0.5% yeast extract. Ten ml BHI agar (Difco) plates were prepared and dried overnight. Each plate was spread with 0.2 ml of the overnight culture then incubated anaerobically in a Gas Pak jar (BBL, Cockeysville, MD) for five days at 10°C. The agar was collected and frozen overnight at -20°C. The agar was allowed to thaw at room temperature (3-4 h) then strained through a double layer of sterile cheesecloth. The cells were removed by centrifugation at 15,319 x g for 10 min. The pH of the supernatant was adjusted to pH 6.5 with 6 N NaOH then filter-sterilized through a 0.45μ AcroDisc (Gelman, Ann Arbor, MI). *L. bavaricus* MN was propagated overnight in APT broth (Difco) at 30°C, the pH adjusted to pH 6.5 with 6 N NaOH and cells removed by centrifugation. *P. pentosaceus* 43200 was propagated overnight in Trypticase soy broth without dextrose (BBL, Cockeysville, MD) plus 0.5% dextrose (Sigma, St. Louis, MO) plus 0.6% yeast extract (Difco) buffered to pH 7.5 with 0.1 M phosphate buffer at 30°C cells removed by centrifugation followed by filter-sterilization through a 0.45μ AcroDisc.

Heat Sensitivity. Crude bacteriocin preparations (1 mL) were aliquoted into Eppendorf tubes and heated at 60°C for 0, 10, 15, 30 and 60 min and at 100°C for 5, 10, 15 and 20

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min. The tubes were placed immediately on ice and assayed for bacteriocin activity by the well-diffusion assay (Rogers and Montville, 1991).

SDS-PAGE. Crude bacteriocin preparations and molecular weight standards with masses of 2512, 6214, 8159, 14404 and 16949 (442472L, BDH Limited, England) were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 20% uniform pore gel as described by Lewus et al. (1992). Gels were stained with silver staining and assayed for activity as described by Lewus et al. (1992).

RESULTS

Mode of Action. Viable *L. sake* 15521 cells could not be recovered from the zones of inhibition produced by *L. plantarum* BN, *L. bavaricus* MN and *P. pentosaceus* 43200. Viable *L. sake* 15521 cells were recovered from control refrigerated seeded plates which had not been exposed to bacteriocin and from the indicator lawn. The addition of protease to the zone of inhibition (protease rescue) did not result in the recovery of the indicator cells.

Optimal pH and Temperature for Bacteriocin Production on Solid Media. The results of the buffered agar assay are presented in Table 1. While zone sizes at comparable pH values vary between media buffered with MES and HEPES, pH optima are evident. For *L. plantarum* BN, little bacteriocin was produced below pH 6.15. For *L. bavaricus* MN, bacteriocin production reached a maximum at pH 6.15-6.5 and tailed off slightly in either direction. For *P. pentosaceus*

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TABLE 1: Bacteriocin production on solid media buffered to various pH values using 0.2 M MES (2-[N-morpholino]-ethanesulfonic acid, or HEPES buffers.

pH (buffer)	<u>L. plantarum</u> BN	<u>L. bavaricus</u> MN zone size (mm)	<u>P. pentosaceus</u> 43200
5.2 (MES)	< 1.0	10.2	5.4
5.8 (MES)	< 1.0	11.4	6.5
6.15 (MES)	< 1.0	13.3	8.0
6.5 (MES)	2.4	14.0	8.2
6.9 (MES)	4.0	10.9	8.1
6.9 (HEPES)	< 1.0	13.1	6.5
7.55 (HEPES)	2.7	10.7	6.6
7.9 (HEPES)	4.0	11.1	6.6

TABLE 2 : Influence of growth temperature on bacteriocin production on solid media.

Strain	Growth temperature			
	4°C	10°C	15°C	30°C
	Zone Size (mm)			
<u>L. plantarum</u> BN	10.0	12.1	13.2	7.0
<u>L. bavaricus</u> MN	13.6	17.3	17.3	21.2
<u>P. pentosaceus</u> 43200	2.2	3.2	2.6	4.5

43200, bacteriocin production appeared equal between pH values from 6.15 - 7.9. Bacteriocin production occurred over the entire pH range (5.2-7.9) measured for L. bavaricus MN and P. pentosaceus 43200.

The affect of growth temperature on bacteriocin production is presented in Table 2. The three organisms produced bacteriocin over the growth temperature range (4°C to 30°C) examined. L. bavaricus MN and P. pentosaceus 43200 produced more bacteriocin with increasing growth temperature. L. plantarum BN produced bacteriocin as follows: 15°C > 10°C > 4°C > 30°C.

Broth Media for Bacteriocin Production. L. plantarum BN produced bacteriocin in APT broth, Trypticase soy broth without dextrose supplemented with 1% glucose and 2% yeast extract, Brain heart infusion broth and Brain heart infusion broth supplemented with 2% yeast extract. However, bacteriocin production in broth media by L. plantarum BN was very variable and inconsistent. L. bavaricus MN produced bacteriocin in all broth media examined. P. pentosaceus 43200 produced bacteriocin only in Trypticase soy broth without glucose plus 1 % glucose and Trypticase soy broth without glucose plus 1 % glucose and 2 % yeast extract.

Determination of Growth Phase of Bacteriocin Production in Broth. The growth curves for L. plantarum BN, L. bavaricus MN and P. pentosaceus 43200 are presented in figures 1a-3a. The production of bacteriocin as measured by the well-diffusion assay are presented in figures 1b-3b. The doubling times, t_d , of L. plantarum BN, L. bavaricus MN and P. pentosaceus 43200 at 30°C were 1.3, 2.1 and 3.9 h, respectively. All three organisms produced the highest "detectable" level of bacteriocin in the early logarithmic phase of growth. After this peak, activity decreased slightly.

Heat Sensitivity. The sensitivity of the bacteriocins to heat treatments at 60°C and 100°C as measured by the well-diffusion assay is presented in Table 3. All of the bacteriocins were more sensitive to the heat treatment at 100°C versus 60°C. The zone size of plantaricin BN was

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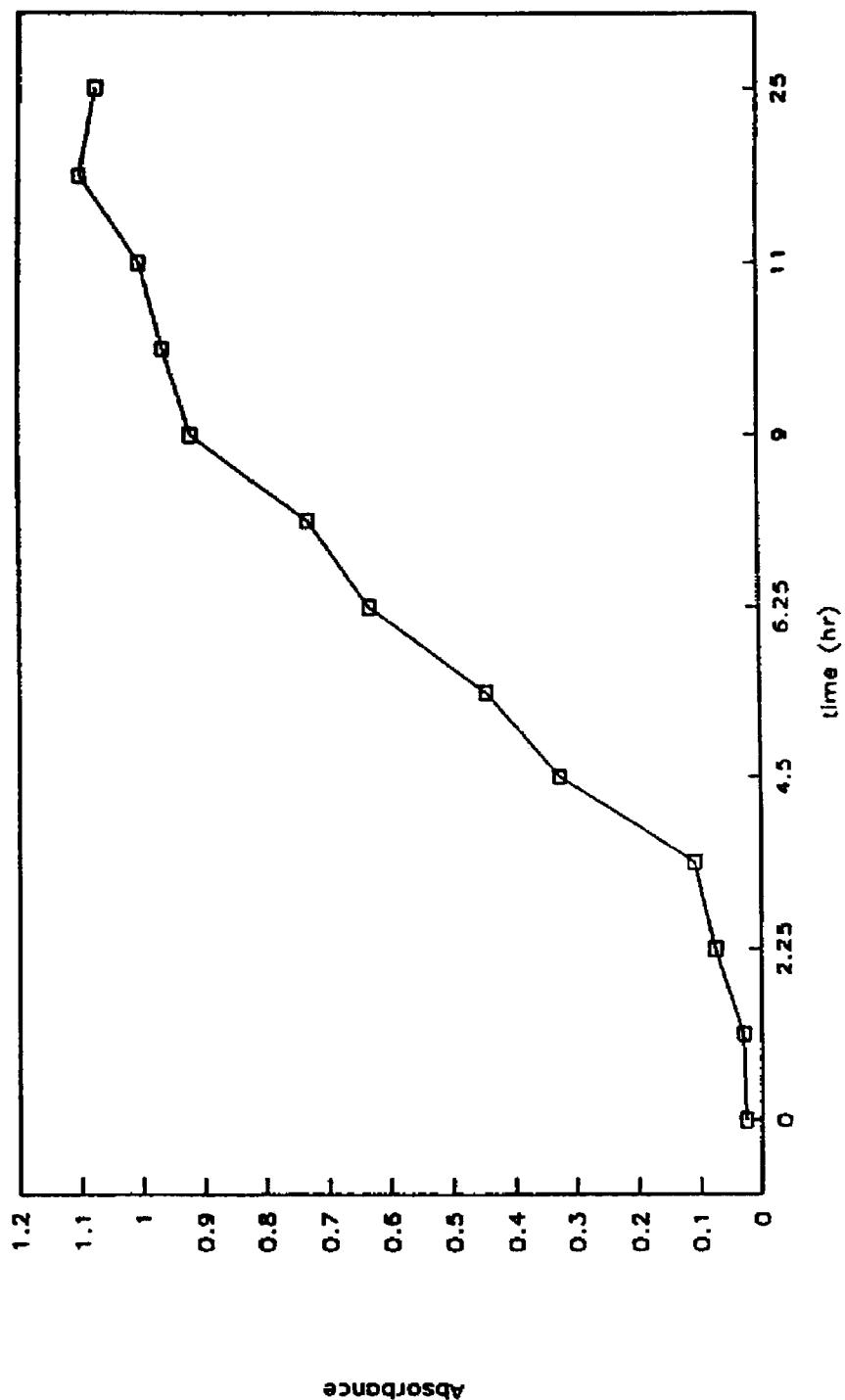


FIGURE 1A

Growth curve of *Lactobacillus plantarum* BN determined in APT
broth at 30°C.

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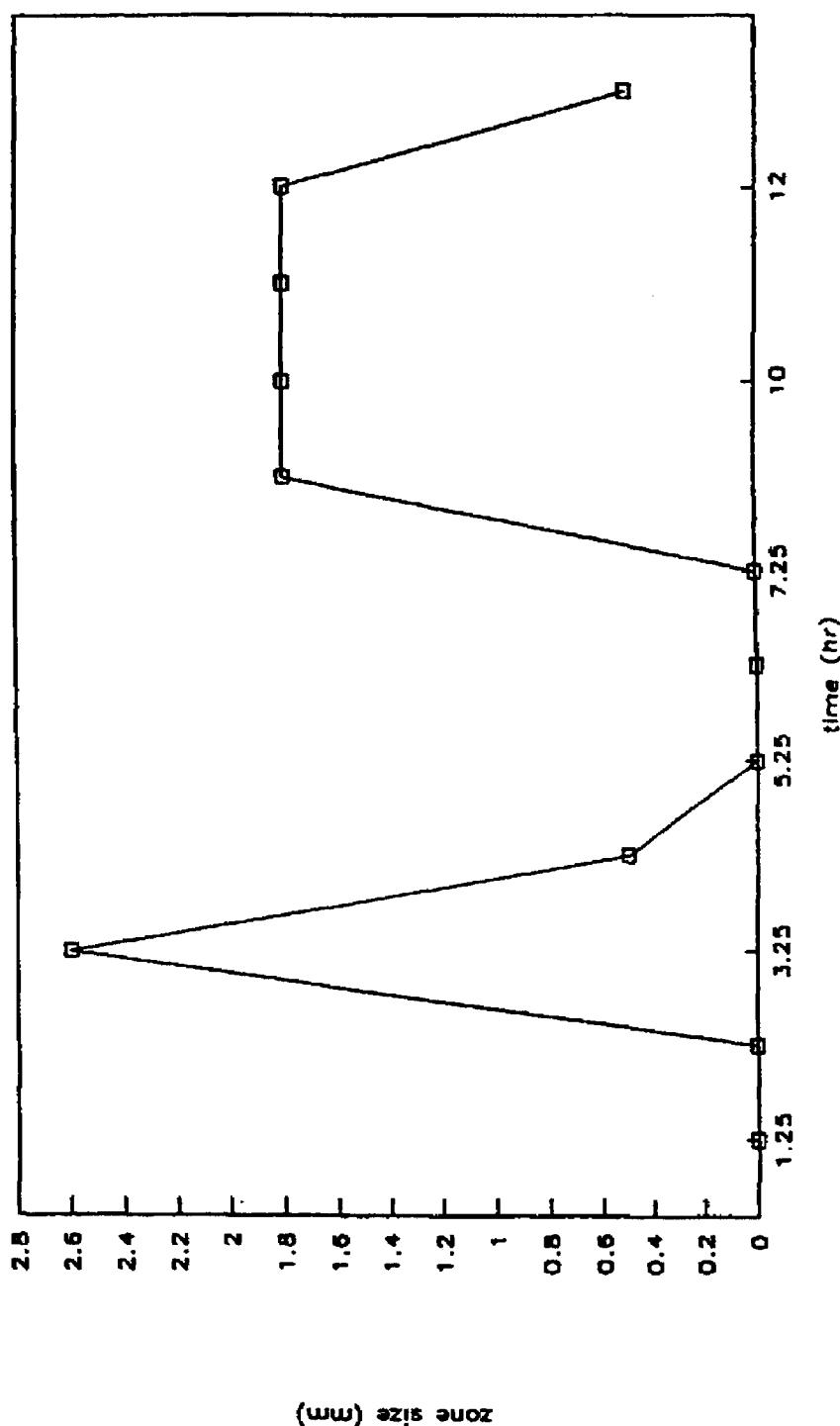


FIGURE 1B

Bacteriocin production with time as determined by the well-diffusion assay for Lactobacillus plantarum BN grown in APT broth at 30°C.

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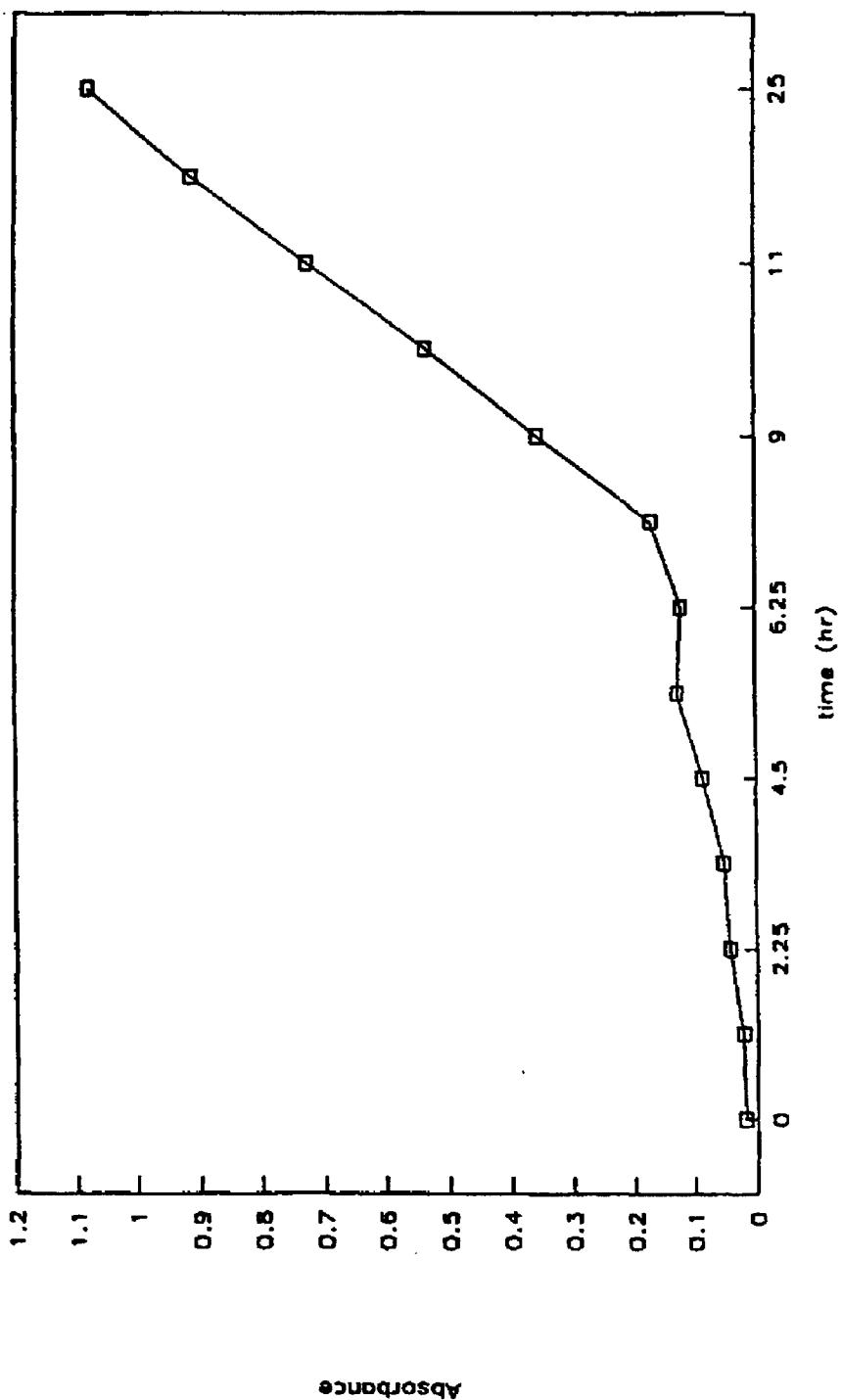


FIGURE 2A
Growth curve of *Lactobacillus bavaricus* MN determined in APT
broth at 30°C.

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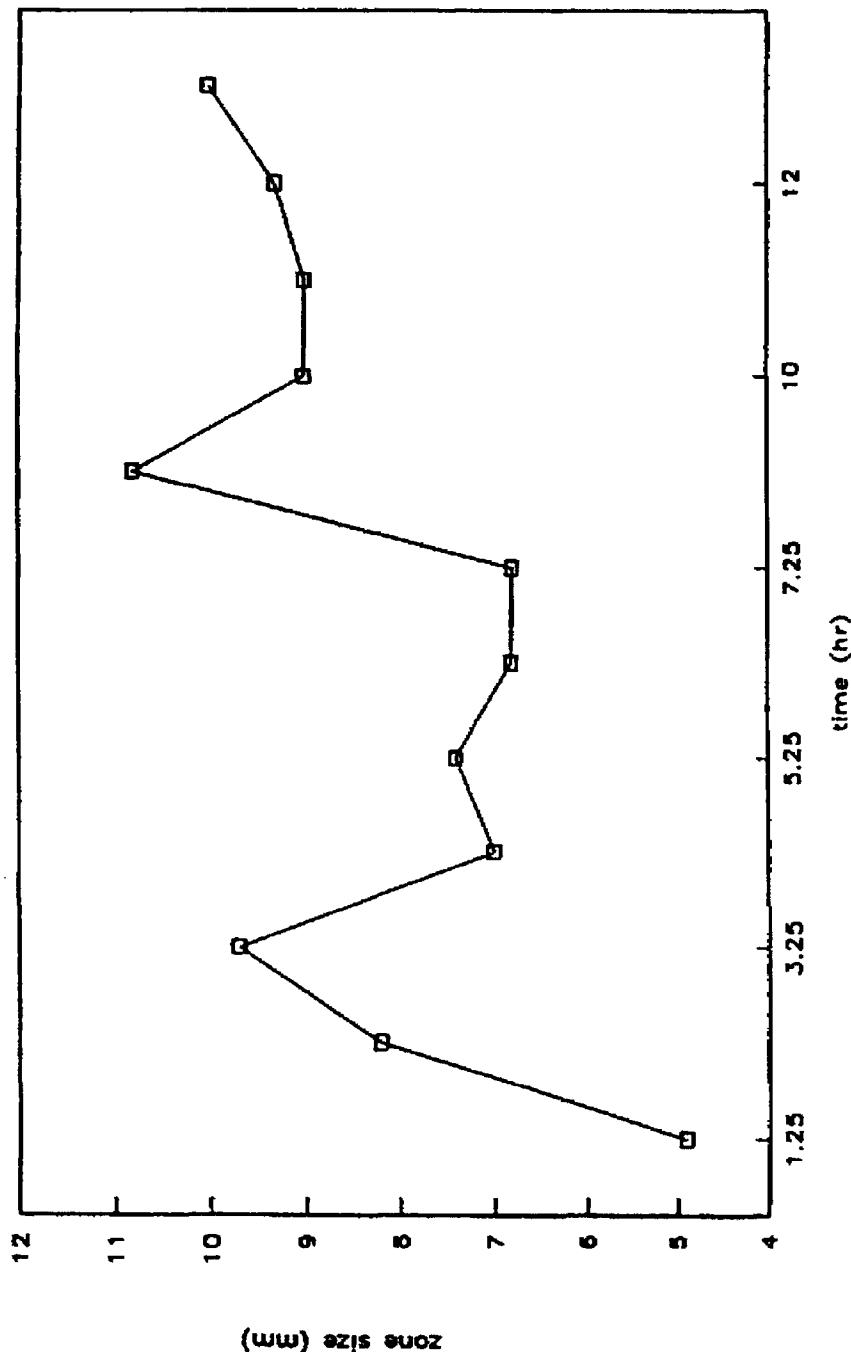


FIGURE 2B
Bacteriocin production with time as determined by the
well-diffusion assay for Lactobacillus bavaricus MN grown in
APT broth at 30°C.

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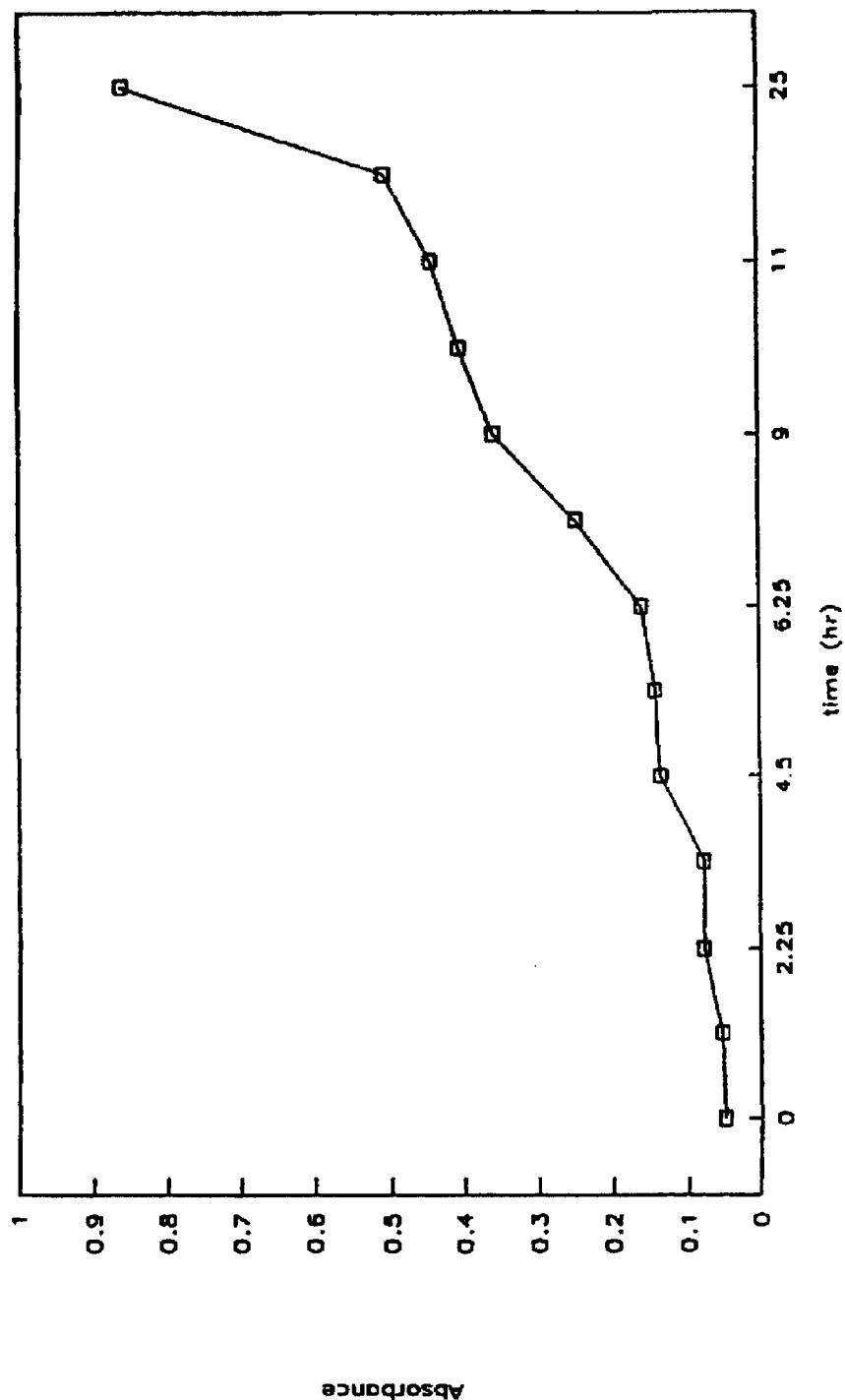


FIGURE 3A

Growth curve of Pediococcus pentosaceus 43200 determined in Trypticase soy broth without dextrose plus 1.0% glucose at 30° C.

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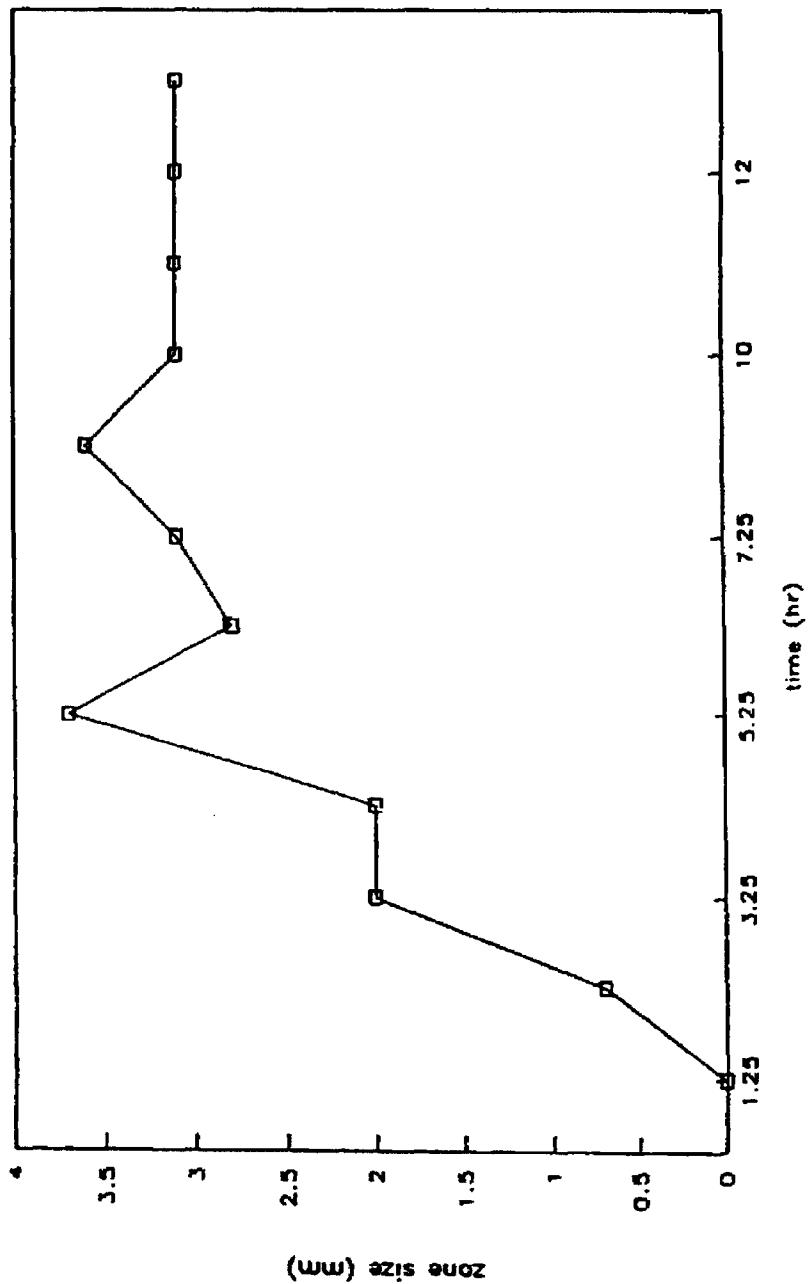


FIGURE 3B

Bacteriocin production with time as determined by the well-diffusion assay for Pediococcus pentosaceus 43200 grown in Trypticase soy broth without glucose plus 1.0% glucose at 30°C.

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TABLE 3: Heat sensitivity of the bacteriocins at 60°C and
100°C as measured by the well-diffusion assay

Temp.	Time (min)	<u>L. plantarum</u> BN	<u>L. bavaricus</u> MN	<u>P. pentosaceus</u> 43200
60°C				
0	2.3	9.2	3.2	
10	1.1	3.0	2.0	
15	1.2	0.0	2.1	
30	<1.0*	0.0	2.0	
60	<1.0	0.0	2.3	
100°C				
0	2.3	9.2	3.2	
5	1.1	4.6	1.5	
10	1.1	0.0	1.0	
15	<1.0	0.0	<1.0	
20	<1.0	0.0	<1.0	

* Zone sizes of <1 are visible but not measurable; 0.0 denotes no visible zone.

reduced 50% by heating at 60°C for 15 min and at 100°C for 10 min. While total activity was not lost, upon further heating only a halo was formed around the well.

The zone size of bavaricin MN was reduced 66% after heating at 60°C for only 10 min and 50% after heating at 100°C for only 5 min. Upon further heating, the activity of the bacteriocin was totally lost. The zone size of pediocin A was reduced 33% after heating at 60°C for 10 min; however, further activity was not lost upon continued heating at 60°C up to 60 min. At 100°C, pediocin A maintained partial activity through 10 min.

SDS-PAGE. When the crude plantaricin BN preparation was electrophoresed on 20% acrylamide gels in the presence of

0.1% SDS, many small bands appeared on the gel along with a thick band of staining near the top of the gel. The portion of the gel overlaid with BHI agar seeded with L. sake 15521 showed no zones of activity. Activity appeared to migrate in one diffuse band corresponding to a molecular weight of 1300 on the portion of the gel sliced into 5 mm portions.

When the crude bavaricin MN preparation was electrophoresed on 20% acrylamide gels in the presence of 0.1% SDS, no activity bands were observed. However, upon silver staining two major bands were seen at MW 9700 and 22,600 as were the molecular weight markers.

When crude pediocin A was electrophoresed on 20% acrylamide gels in the presence of 0.1% SDS, activity was detected on the portion of the gel overlaid with BHI agar seeded with L.sake 15521 and corresponded to a MW 11,200. On the portion of the gel cut into slices, activity was very diffuse throughout the gel. When silver stained, pediocin A showed diffuse staining with somewhat darker staining in the MW range of 10,000 to 11,000.

DISCUSSION

Plantaricin BN, bavaricin MN and pediocin A all demonstrated a cidal mode of action as determined by the plate diffusion assay.

The optimal pH for production on solid media was influenced by the growth of the organism as well as the activity of the bacteriocin. The production of plantaricin BN was favored in a slightly alkaline environment while the production of bavaricin MN was favored in an acidic

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environment and that of pediocin A was favored in a slightly acidic to alkaline environment. Nisin solubility and activity is quite high at low pH and quite low at high pH (Hurst, 1981; Liu and Hansen, 1990). In general, the variability of pH optima appears to reflect the growth of the organisms as well as the activity and solubility of the bacteriocins. However, high cell mass does not insure high bacteriocin production. Biswas et al. (1991) demonstrated that both a low final pH and large cell mass are necessary for a high level of pediocin AcH production. Thus, it is clear that no generalization about optimum conditions for bacteriocin production can be made; the conditions must be determined experimentally for each specific bacteriocin.

Growth temperature not only influences production of bacteriocin by the microorganism but the growth and amount of growth by the microorganism. It might be argued that bacteriocin production occurred at 30°C (the incubation temperature of the indicator) and not at the lower temperatures. If this were true, one would expect no difference in the zone sizes of cultures grown at different temperatures. This was not the case, and one strain, L. plantarum BN, bacteriocin production was less at 30°C than at did lower temperatures. Okereke and Montville (1991) were recently able to isolate bacteriocin produced by this strain at 4 and 10°C and demonstrate its activity in a deferred antagonism assay.

While plantaricin BN, bavaricin MN and pediocin A were all produced in broth media, the conditions for production

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varied considerably. Difficulty in obtaining bacteriocin activity in broth is not a novel finding (Mayr-Harting et al., 1972). Plantaricin BN did not produce bacteriocin consistently or in great amounts. As with some other gram-positive bacteriocins (Tagg et al., 1976), it was necessary to prepare culture extracts from plates to get consistent activity for plantaricin BN. The inability of Roller et al. (1990) to detect antimicrobial activity associated with P. pentosaceus 43200 was due to their use of a broth medium which did not support pediocin A production.

The production of bacteriocins in the exponential phase of growth agreed with results obtained for bacteriocins produced by other lactic acid bacteria (Bhunia et al., 1987; Geis et al., 1983; Hastings and Stiles, 1991; Muriana and Klaenhammer, 1987).

Little information was obtained from the electrophoresis of crude plantaricin BN on SDS-PAGE. The very diffuse, faint band of activity around MW 1300 could be attributed to bromophenol blue in the gel. However, based on its relative heat sensitivity coupled with its relative insensitivity to protease inactivation (Lewus et al., 1992) as well as the silver staining banding pattern on the SDS-PAGE gel, the molecular weight of plantaricin BN is probably higher than 10,000. Preliminary work with centricron 10 ultrafiltration units (Amicon, Beverly, MA) also suggest that the molecular weight of plantaricin BN was greater than 10,000.

The sensitivity of bavaricin MN to proteases (Lewus et al., 1991), heat and SDS denaturation, the observance of two major bands on SDS-PAGE upon silver staining, preliminary work with centricon 10 units indicate that bavaricin MN is a high molecular weight bacteriocin corresponding to MW 22,600. There are no other reported bacteriocins produced by *L. bavaricus* in the literature.

Based on the results of the SDS-PAGE, the molecular weight of pediocin A appeared to be approximately 11,000. The presence of diffuse bands upon silver staining of the pediocin A lane indicates that either not enough pediocin A was present to be detected or that the pediocin A does not silver stain well. Barefoot and Klaenhammer (1984) obtained similar results with lactacin B.

The varying characteristics of these three bacteriocins and their spectrum of activities suggests that their use to insure the safety of minimally processed refrigerated meat products may be feasible. Further purification of these bacteriocins to discern their characteristics as well as inoculated meat studies employing the bacteriocins deserve further research.

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